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13. ABSTRACT *(Maximum 200* Thrombospondin-1 (TSP1) is a modular trimeric protein with several documented functions, including its role as an angiogenic inhibitor. TSP1, TSP1 fragments, and certain TSP1 conserved peptide sequences have been shown to exert an endothelial-specific inhibition of growth and migration. Peptides derived from conserved sequences within the type 1 repeats of TSP1 have been shown to block neo-vascularization *in vivo*, inhibit DNA synthesis, and migration of cultured endothelial cells *in vitro*. Our laboratory has shown that recombinantly expressed human TSP1 (hTSP1) type 1 repeats inhibit migration of bFGF stimulated bovine adrenal microvascular endothelial cells. This study seeks to define the structural basis for the angiostatic effect of the hTSP1 type 1 repeats. I will employ biophysical methods in a comparative study of TSP1 type 1 repeats and active peptides based on type 1 sequences. I have successfully generated recombinant baculoviruses that express the three type 1 repeats in tandem (P123) and the third type 1 repeat (P3) as histidine-tagged fusion proteins. A purification scheme for the recombinant proteins including removal of the histidine-tag has been established. To date, N-terminal sequencing, carbohydrate analysis, and circular dichroism have been performed.

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Kristin J. Heuvel 8/28/97

PI - Signature Date

IV. Table of Contents

V. Introduction

Thrombospondin-1 (TSP1) is a member of a family of modular glyco-proteins including thrombospondin-2 (1), thrombospondin-3 (2), thrombospondin-4 (3), cartilage oligomeric matrix protein (4), and F-spondin (5). TSP1 is a disulfide-bonded trimer with an approximate molecular mass of 450KDa. The modular structure of trimeric TSP1 is illustrated in figure 1 and is based in part on the electron microscopy of rotary shadowed TSP1. The amino-terminus consists of a globular heparin-binding domain (6) and is followed by the central stalk region. The 70KDa central stalk region contains three types of domains which are also found in other proteins, these include a procollagen module, three properdin or type 1 repeats, and three EGF-like or type 2 repeats (6,7). The C-terminus of TSP1 is dependent on Ca^{++} for its structural integrity (7,8) and it contains the cell adhesion motif RGD.

TSP1 is synthesized and secreted by various cell types where it can become incorporated into the extracellular matrix (ECM). The ECM is an important modulator of cell proliferation, migration, and differentiation (9-12). Several investigations indicate that TSP1 is a normal component of the extracellular matrix of mammary tissue. The level and pattern of expression of TSP1 is altered by the developmental (13), lactational (13), and neoplastic (14) state of the mammary tissue. TSP1 is found deposited in the basement membrane of normal breast tissue (14) and is also found in breast milk (15). The expression of TSP1 is altered in neoplastic breast tissue. In contrast to normal breast tissue, increased levels of TSP1 was present in the basement membrane surrounding preinvasive tumors while TSP1 was absent along the progressing front of invasive ductal carcinomas (14). *In vitro* experiments have demonstrated that an increase in TSP1 mRNA and protein expression correlated with a decrease in malignant progression (16) and inhibition of metastases (17).

Metastasis of tumor cells is a multi-step process that includes angiogenesis. A reasonable hypothesis suggested to explain the inhibition of metastases and malignant progression is that the presence of TSP1 in the tumor micro-environment modulates an angiostatic effect (18-21). This idea is supported by a growing body of work that has shown TSP1, TSP1 fragments, and certain TSP1 conserved peptide sequences to exert an endothelial-specific inhibition of growth and migration.

Our lab is interested in further examining the cell-specific effect of TSP1 by localizing active regions of the molecule and cell surface receptors that interact with TSP1. Using the baculovirus system we have recombinantly expressed intact as well as portions of TSP1 such as the type 1 repeats. Our lab has shown that recombinantly expressed human TSP1 (hTSP1) type 1 repeats does inhibit migration of bFGF stimulated bovine adrenal microvascular endothelial cells. This study seeks to define the structural basis for the angiostatic effect of the hTSP1 type 1 repeats.

Each hTSP1 monomer contains three type 1 repeats that are encoded for by individual exons (22) and which contain approximately sixty amino acids. An alignment of the three type 1 repeats in TSP1 is shown in figure 2. I will employ several biophysical methods in a comparitive study of hTSP1 type 1 repeats and active peptides based on the type 1 sequences. I will utilize circular dichroism, fluorescence spectroscopy, and X-ray crystallography. Large quantities, tens of milligrams, of highly purified protein is necessary for these experiments. I have expressed the three type 1 repeats of human TSP1 in tandem (P123) and the third type 1 repeat alone (P3) using the baculovirus system to meet these ends. Determination of the structure critical for activity could be used as a starting point for the design of small molecules which elicit the same function.

VI. Body

A. Criteria for Design of Protein Expression System

Baculoviruses are a group of viruses that contain circular, double-stranded genomic DNA and are capable of infecting insect cells. The baculovirus system was chosen due to its ability to catalyze disulfide bond formation, promote post-translational modifications, and produce large quantities of recombinant proteins. Our lab has expressed various combinations of the type 1 repeats as fusion proteins with the gelatin-binding domain of fibronectin. These recombinant fusion proteins, termed GELEX fusions, allow for the affinity purification of the recombinant proteins on gelatin sepharose. I initially used the GELEX system to express and purify recombinant type 1 repeats. However, upon complete characterization of this system, I found it to be unsuitable for the isolation of very pure and homogeneous recombinant protein that I required for biophysical studies. Further details of the problems encountered is found in the Recommendations section of the Body. A new fusion construct to express the type 1 repeats in the baculovirus system was then sought.

A decision to express the type 1 repeats as a fusion protein with a series of six histidines (His-tag) was made. The use of His-tags for affinity purification is commonly used by labs, including those performing biochemical and biophysical studies. In addition, histidine tagged proteins have been successfully expressed in the baculovirus system and baculovirus transfer vectors containing these sequences are commercially available. There were three things I required of the baculovirus transfer vector. First, it must contain a signal sequence that would direct the recombinant protein into the endoplasmatic reticulum. Since each type 1 repeat is proposed to contain three disulfide bonds and the third repeat has an N-linked glycosylation sequence (N-X-S/T), the recombinant protein must pass through the secretory pathway. Second, I wanted the His-tag at the C-terminus of the recombinant molecule. The baculovirus system is a dying one and the possibility to obtain recombinant protein with

premature termination exists. Therefore, placement of the His-tag at the C-terminus ensures that full length protein is selected for in the purification. The third requirement for the baculovirus vector stemmed from the desire to remove the His-tag from the purified recombinant protein. Since one of the goals of this project is to obtain diffraction quality crystals, I wanted as little extra coding sequence that might interfere with the crystallization process. There are, however, reports in the literature of crystals being obtained for His-tagged proteins. To remove the His-tag, I needed a protease site encoded 5' to the His-tag. Unfortunately , there were no commercially available baculovirus transfer vectors that incorporated these features. Therefore, the pCOCO baculovirus transfer vector was constructed based on these specifications.

B. Experimental Methods

Construction of pCOCO Baculovirus Transfer Vector

The pAcGP67A baculovirus transfer vector (Pharmingen) was chosen as the starting point. It contains the GP67 signal sequence 5' to the multiple cloning site (MCS). This signal sequence is under the control of the very strong polyhedrin promoter. The pAcGP67A vector was modified 3' to the MCS by the addition of a DNA sequence that encodes a thrombin cleavage site followed by a His-tag. The exact sequence for the cleavage site is shown in Figure 3A. A PstI restriction site was incorporated between the coding region for the thrombin cleavage site and the His-tag.

The primers used to generate this fragment are shown in Figure 3B and are called COCO forward and COCO reverse. These two primers have a nineteen base pair overlap. They were denatured at 94C and then allowed to anneal. Extension was accomplished with Deep Vent DNA polymerase (New England Bioloabs) at 70C for 7 minutes. The fragment was purified and then digested with XbaI and PpuMI. The digested product was purified and ligated into the pAcGP67A MCS at the XbaI and PpumI sites. The resulting transfer vector is termed pAcGP67.COCO or pCOCO (Figure 4).

Cloning hTSP1 Type 1 Repeats into pCOCO Baculovirus Transfer Vector

The sequences encoding P123 and P3 were amplified from hTSP1 cDNA by the polymerase chain reaction (PCR). The forward and reverse primers for P123 as well as the forward primer for P3 amplification are shown in Figure 5. The reverse primer used to amplify P3 was the same as used for P123. The forward primers introduced an XmaI site while the reverse primer added an XbaI site. These PCR products were cloned into the XmaI and XbaI sites in the MCS of pAcGP67.COCO. The DNA sequences were verified for all constructs prior to the generation of recombinant baculoviruses.

Generation of Recombinant Baculoviruses

Recombinant baculoviruses were generated using Baculogold (Pharmingen) linearized AcNPV viral DNA. Co-transfections into Sf9 cells with baculogold and P123.COCO or P3.COCO were performed using CellFectin (Gibco-BRL). Recombinant baculoviruses were cloned by plaque purification. High titer (1-5x10^8 pfu/ml) virus stocks were prepared using Sf9 cells.

Purification of Type 1 Repeats

High 5 cells (BTI-TN-5B1-4) were grown at 27C in spinner flasks using SF900 II serum free media. For large scale production, one liter spinner flasks were used. Cells were infected at a density of 1x10^6 cells/ml. A multiplicity of infection (MOI) of 5 was routinely used and the infection was allowed to proceed for 60-62 hours.

Since the P123.COCO and P3.COCO are directed to the insect secretory pathway by the GP67 signal sequence, the first step in the purification procedure involves clarifying the conditioned media (CM). The insect cells are pelleted at ~50-80xg for 10 minutes . The CM is carefully decanted from the cell pellet and PMSF (Sigma) is added to a final concentration of 2mM. To remove cell debris, the CM is centrifuged at ~20,000xg for 15 minutes and then the supernatant is removed.

The second step involves incubation of the clarified CM in batch with NiNTA resin (Qiagen) using a suspended paddle/stir bar rotating at ~10rev/min for 2 hours. The amount of resin to use is determined by the expression levels of the fusion protein.

The third step of washing and eluting the protein from the NiNTA resin was then performed. The media was carefully removed from the settled resin by aspiration. The resin was transferred to 50ml conicals and gently pelleted at ~30xg for 3 minutes. The CM was removed, the NiNTA was washed one time with Tris-buffered saline (TBS) pH 7.4 and then transferred to a column. The flow was adjusted to 0.5ml/min and allowed to rinse with TBS until baseline was reached. The column was then washed with a 10mM TBS, 10mM imidazole pH 7.4 solution until a new baseline was reached. The column was eluted with TBS, 250mM imidazole pH 7.4. The fusion protein is eluted within the first four column volumes with the majority of fusion in the second column volume.

The fourth step in the purification of the recombinant protein involves removal of the His-tag by proteolytic cleavage with thrombin. The fractions from the NiNTA column were pooled and dialyzed at 4C against thrombin reaction buffer (50mM Tris-Cl, 150mM NaCl, 2.5mM $CaCl_2$, pH 8.5). The conditions necessary to remove the His-tag using biotinylated-thrombin (Novagen) were determined. An 18-20 hour digestion at room temperature using ~4munits thrombin/ug of fusion was sufficient. The biotinylated thrombin was removed using Streptavidin-agarose (Novagen) according to manufacturer's instructions. In order to remove

any incompletely digested fusion protein, the sample was incubated with NiNTA resin in batch; it was separated from the resin by pouring through a column. The resulting sample was adjusted to 0.02% sodium azide and 2mM Pefabloc SC (Boehringer Mannheim). The recombinant protein was dialyzed into TBS at 4C and subsequently concentrated using a Centriplus-3 ultrafiltration device (Amicon).

N-terminal Sequencing of P123.COCO

Purified P123.COCO was denatured, reduced, and run on a 14% SDS-PAGE gel. The proteins were transferred to PVDF and the blot was stained with 0.1% Amido Black. The N-terminal sequencing was performed in the lab of Dr. Johan Stenflo, Lund University, Sweden. The sample underwent 15 cycles of sequencing.

Glycosylation of P123

The DIG Glycan Detection (Boehringer Mannheim) system has been used to determine if baculovirally expressed P123 is glycosylated. Manufacturer's instructions for detecting glycosylation of immobilized proteins were followed. Both P123 expressed as COCO or GELEX fusions were tested. The GELEX constructs had been treated with trypsin to cleave the P123 from the GELEX portion.

Initial Characterization of P3 by Circular Dichroism

The University of Wisconsin-Madison Biophysics Instrumentation Facility's AVIV 62 ADS circular dichroism spectrophometer was used to monitor the far-UV CD signal for P3. The P3 sample was 0.19ug/ml in 10mM Potassium Phosphate, 100mM Sodium Chloride, pH 7.3 and was placed in a quartz cuvette of pathlength 0.1cm. A CD spectra was obtained by scanning from 260nm to 195 nm at 25C. The spectra of the buffer alone was subtracted from that of the sample. A temperature scan from 25C to 70C was performed on the P3 sample. The CD signal at 229nm and the total fluorescence emmission when exciting at 291nm was monitored. The temperature was increased in 5C increments using a slope of 50C/min and an equilibration time of 1minute.

C. Results and Discussion

Construction of pCOCO Baculovirus Transfer Vector

A graphical map of the pCOCO baculovirus transfer vector is shown in Figure 4. The cDNA for P123 and P3 were cloned into the XmaI and XbaI sites as described in the Experimental Methods section. The resulting baculovirus transfer vector can be used to generate recombinant baculoviruses that express the cDNA as a fusion protein. The fusion protein will be directed to the secretory pathway by the amino terminal GP67 siganl sequence. The carboxy-terminus of the fusion protein (COCO) contains a thrombin cleavage

site and a series of six histidines. The His-tag allows the recombinant protein to be readily purified on nickel-chelate resin while the thrombin cleavage site allows the His-tag to be subsequently removed.

Purification of Type 1 Repeats

Yields of 20-50ug of fusion per milliliter of conditioned media were obtained for P123.COCO. The results of SDS-PAGE gel and Western blot of P123.COCO is shown in Figure 6. In general, the level of expression for P3.COCO was lower than that obtained for P123.COCO when the same infection conditions were employed. A time course of infection using two different MOIs revealed that the conditions originally used, MOI=5 for ~62 hrs, yielded the best results. Figure 7 shows the results of this time course study. In general, the fusion protein is eluted from the NiNTA column within the first four column volumes with the majority of fusion in the second column volume (figure 8). The conditions necessary to remove the His-tag using biotinylated-thrombin (Novagen) were determined. An 18-20 hour digestion at room temperature using ~4munits thrombin/ug of fusion was sufficient. In general, the P3.COCO digestion was nearly complete while the P123.COCO was ~90% complete (figure 9).

N-terminal Sequencing of P123.COCO

The COCO fusion proteins are expressed with the 38 mino acid long GP67 signal sequence. In order to determine if the fusion proteins had the GP67 signal sequence removed at the predicted cleavage site and to determine whether the signal sequence site was homogeneous, N-terminal sequencing of P123.COCO was initiated. The P123.COCO sample was determined to have a homogeneous N-terminus beginning at the anticipated amino acid, based on the predicted signal sequence cleavage site. The results are presented in Figure 10.

Glycosylation of P123

The third type 1 repeat of human TSP1 contains a site (N-X-S/T) for N-linked glycosylation. The DIG Glycan Detection system has been used to determine if baculovirally expressed P123 is glycosylated. The three step method employs an enzyme immunoassay to detect sugars on immobilized protein. The result of this analysis shows that P123 expressed in either the COCO or GELEX expression systems is glycosylated. The upper band in the P123.GELEX lane is the GELEX portion of the fusion. The size of P123 expressed in GELEX is slightly larger thean P123 expressed as a fusion with COCO due to extra coding sequence present in GELEX. The lower band is most likely the second and third type 1 repeats. The region linking the first and second type 1 repeats is trypsin sensitive.

Initial Characterization of P3 by Circular Dichroism

A CD spectra was obtained by scanning from 260nm to 195 nm at 25C. The spectra of

the buffer alone was subtracted from that of the sample. The plot is shown in Figure 12. It is marked by the positive ellipticity above ~202nm. The protein properdin contains six TSP1 type 1 repeats which compose ~80% of the primary sequence. The far-UV CD spectrum of properdin (23) is positive above ~195nm and has a similar shape to that of P3.

A temperature scan from 25C to 70C was performed on the P3 sample. The CD signal at 229nm and the total fluorescence emmission when exciting at 291nm was monitored. As seen in Figure 13, the CD signal monitored at 229nm decreased with increasing temperature with the chage beginning between 45-50C. Conversely, the total fluorescence of the sample increased with increasing temperature. The first significant change occurred between 45-50C. After returning the sample to 25C, a second far-UV CD spectrum was obtained. The spectrum before heating (blue) and the spectrum after heating (red) are overlayed in Figure 14. We can see the change that occurred in heating the sample to 70C was reversible.

D. Recommendations in Relation to Statement of Work

The original project detailed the expression of hTSP1 type 1 repeats as a recombinant fusion protein with the gelatin-binding domain of fibronectin (GELEX fusions). Many problems were encountered in obtaining large quantities of highly purified material. Expression of the recombinant fusion was ~10ug/ml; however, approximatley 60% of the mass was due to the presence of the gelatin binding domain which served as the fusion partner and allowed purification of the recombinant protein on Gelatin-Agarose. The second problem encountered was the removal of the gelatin-binding domain. Although I determined conditions to cleave >95% of the the fusion protein without cleaving in between the modules, the removal of the contaminating gelatin-binding domain proved problematic. There was a small population that would not rebind to gelatin agarose following trypsinization. Although other types of chromatography were investigated to remove the contaminating protein, the main problems encountered were very low yields and the formation of multimers of the type 1 repeats. Proteins expressed as fusions in the GELEX system retain a cross-linking site following removal of the gelatin-binding domain with trypsin. The presence of multimers was noted for different proteins expressed as fusions in the GELEX system and is thought to be due to this cross-linking site. In addition, the type 1 repeats following trypsinization to remove the gelatin-binding domain always appeared as two closely spaced bands on a reducing SDS-PAGE gel and Western blots. The differences between the two populations was not due to differences at the amino-terminus as determined by N-terminal sequencing. In addition, neither band appeared to be gylcoysylated as determined by the DIG Glycan detection described in the methods section. Therefore, the origin of the two bands could not be attributed to differential signal sequence cleavage or glycosylation. The biophysical

studies I have proposed require large amounts of very pure protein. It became apparent that protein of this quantity and quality was not readily obtained with the GELEX system. As explained in section VI.A, expression of the type 1 repeats as His-tag fusions using the baculovirus system was initiated.

The problems encountered with the GELEX protein expression system and the initiating of a new method of protein purification has slightly set back my progress. I am completing the initial characterization of the type 1 repeats and am now ready to begin technical objective 2, secondary structure prediction. In addtion, I will now synthesize the two biologically active peptides so that comparisons between recombinant type 1 repeats and the peptides can be made. In addition, I am also ready to begin the initial fluorescence studies to probe the conformational environment of the conserved tryptophans. Under the guidance of Dr. Ivan Rayment, I had previously set-up a crystallization survey utilizing protein obtained from the GELEX system. Since the purity of the protein was less than optimal and since the protein appeared as two bands on reducing SDS-PAGE, we were skeptical of the ability to obtain crystals. Hanging-drop vapor diffusion using 102 different solutions at room temperature and 4C was set-up, but neither yielded crystals. I am working to obtain sufficient quantities of protein to start another crystallization survey.

VII. Conclusions

A new baculovirus transfer vector has been constructed that includes a signal sequence 5' to the multiple cloning site (MCS) and a sequence encoding a thrombin cleavage site followed by a series of six histidines 3' to the MCS. In-frame cloning of the cDNA into the MCS and subsequent generation of recombinant baculoviruses, allows its expression and secretion into the culture media as a histidine-tagged fusion protein. The histidine-tag allows the recombinant protein to be readily purified by nickel-chelate chromatography. The tag can be removed by cleavage of the protein with thrombin. This system has allowed good expression (10-50ug fusion/ml culture media) of hTSP1 type 1 repeats and a means to obtain pure protein. The N-terminus of the P123.COCO fusion protein was cleaved at the anticipated site and is homogeneous. P123.COCO appears to be glycosylated, as anticipated. Determination of the glycosylation state of P3.COCO is in progress, as well as if the glycosylation is N-linked. Initial spectroscopic characterization of a single type 1 repeat by circular dichroism has begun. The CD spectra above 195nm resembles that of the protein properdin which contains six type 1 repeats. The preliminary characterization of the type 1 repeats is progressing and a more thorough investigation into the structure and stability of these modules can now begin.

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Figure 1: Trimeric Organization of TSP1

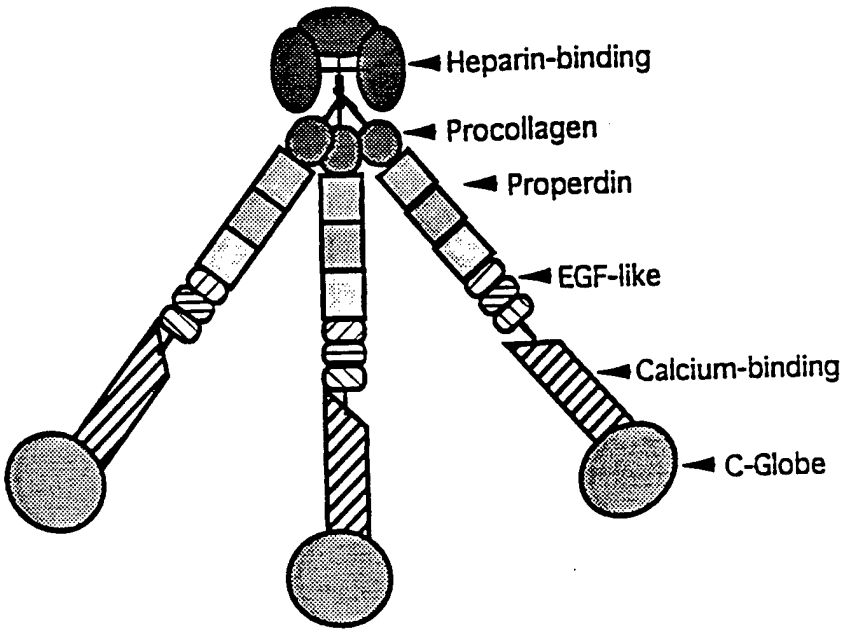


Figure 2: Human TSP1 Type 1 Repeats

```
1st   SDSADDG WSPWSEW TSC STSC GNGIQQ RG RSC DSLNNR.....C EGSSVQTRTC HIQEC DKRF
2nd     KQDGG WSHWSPW SSC SVTC GDGVIT RIRLC NSPSPQMNGKPC EGEARETKAC KKDAC PI
3rd       NGG WGPWSPW DIC SVTC GGGVQK RSRLC NNPTPQFGGKDC VGDVTENQIC NKQDC PID
```

Figure 3 A: Region Added to pAcGP67 to Construct pCOCO

		Thrombin Site								Histidine-Tag						
Protein	L	E	L	V	P	R	G	S	A	A	G	H	H	H	H	H	H	Z
DNA	ctt cta gaa tta gtg cct cgc gga agc gct gca ggg cat cac cat cac cat cac tag gac cta ct																	
	XbaI									**PstI**							**PpumI**	

Figure 3B: Primers used to Construct pCOCO

COCO Forward	5' ctt cta gaa tta gtg cct cgc gga agc gct gca ggg cat cac c 3'
COCO Reverse	5' ag tag gtc cta gtg atg gtg atg gtg atg ccc tgc agc gct tcc 3'

Figure 4: Features of Baculoviral Transfer Vector pCOCO

|----------Proposed GP67 signal sequence----------|

Protein MLLVNQSHQGFNKEHTSKMVSAIVLYVLLAAAAHSAFA A D P G

DNA gcg gat ccc ggg

BamHI XmaI

Thrombin Site **Histidine-Tag**

Protein L E L V P R G S A A G H H H H H H Z

DNA ctt cta gaa tta gtg cct cgc gga agc gct gca ggg cat cac cat cac cat cac tag gac cta ct

XbaI **PstI** **PpumI**

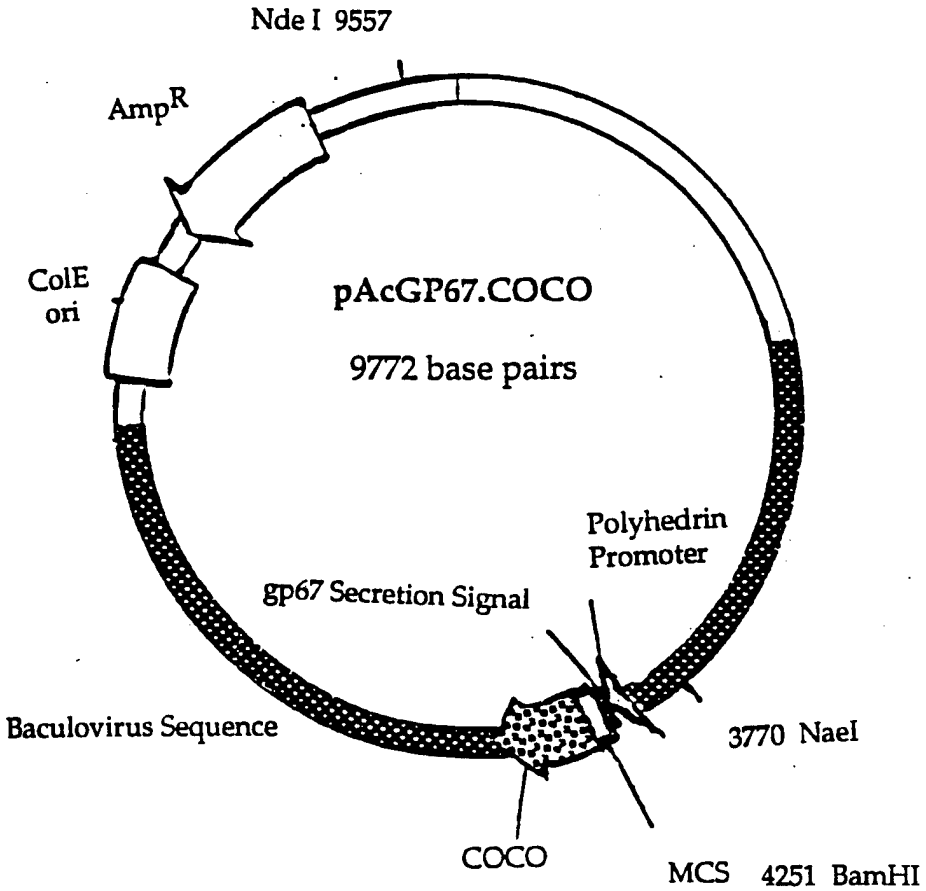
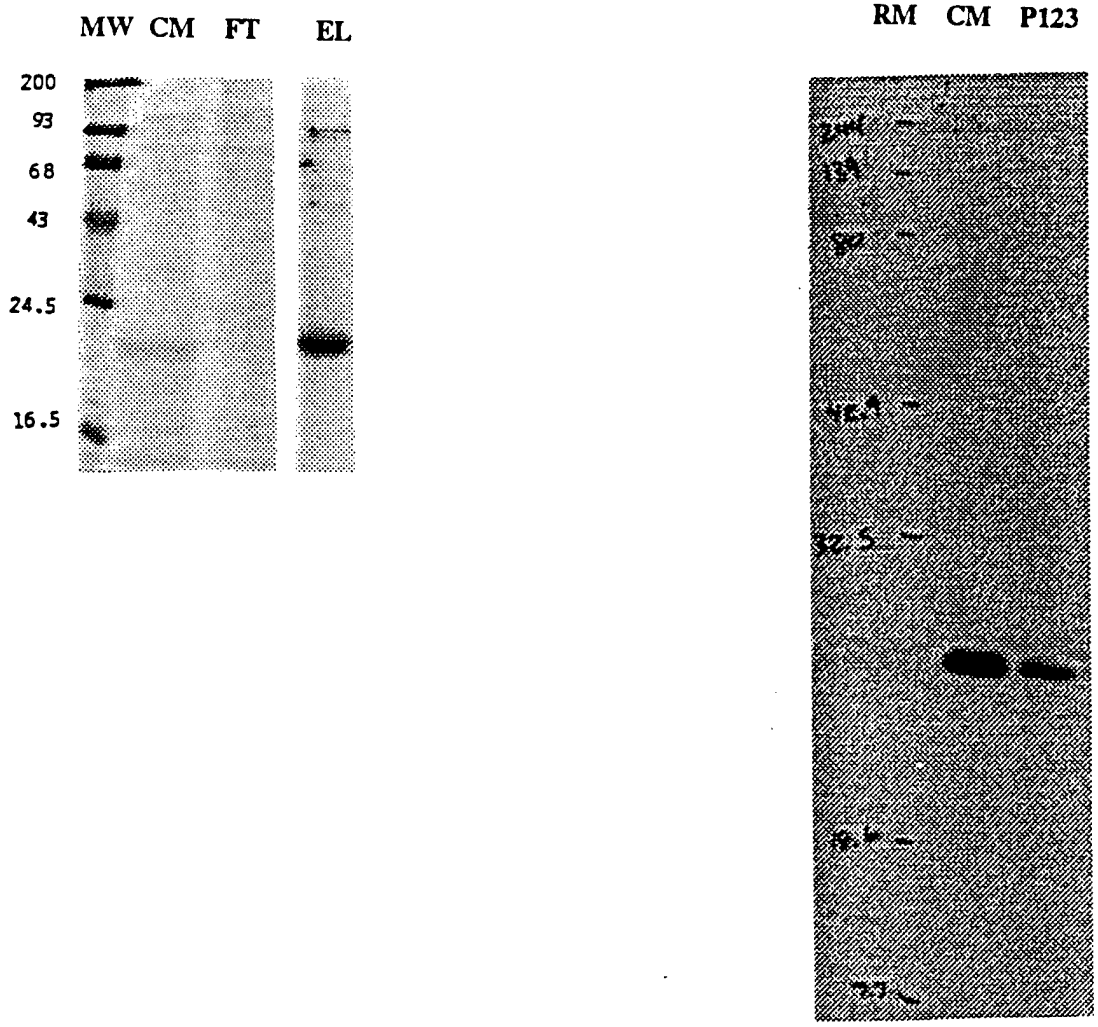


Figure 5: Primers used to Amplify hTSP1 Type 1 Repeats

P123 Forward	5' tcc ccc ggg agc gac tct gcg gac gat gg
P123 Reverse	5' ggg tct aga att gga cag tcc tgc ttg ttg c
P3 Forward	5' tat ccc ggg atc aat gga ggc tgg ggt cct tgg

Figure 6

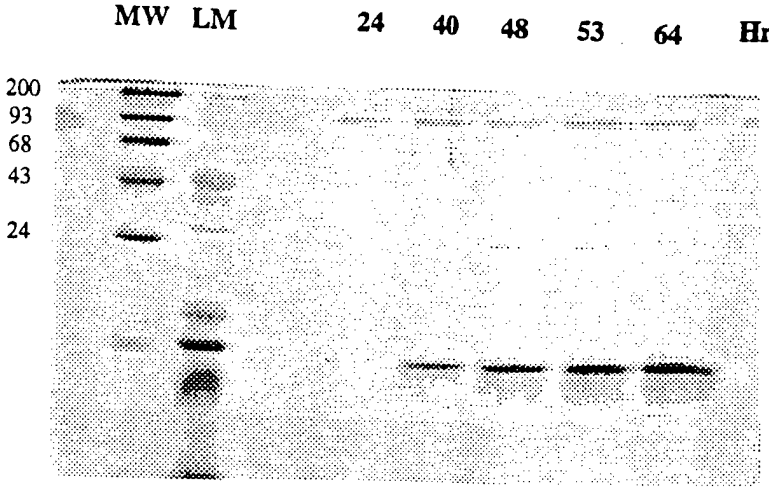


A) Expression of P123.COCO in the baculovirus system. Samples were denatured with SDS, reduced with BME, and boiled for 5 minutes prior to loading on a 12% SDS-PAGE. MW, Molecular weight markers; CM, 40ul Clarified conditioned media, pre-Ni-NTA; FT, 40ul CM after incubation with Ni-NTA resin; EL, Eluate from the Ni-NTA resin after incubation with 200ul CM.

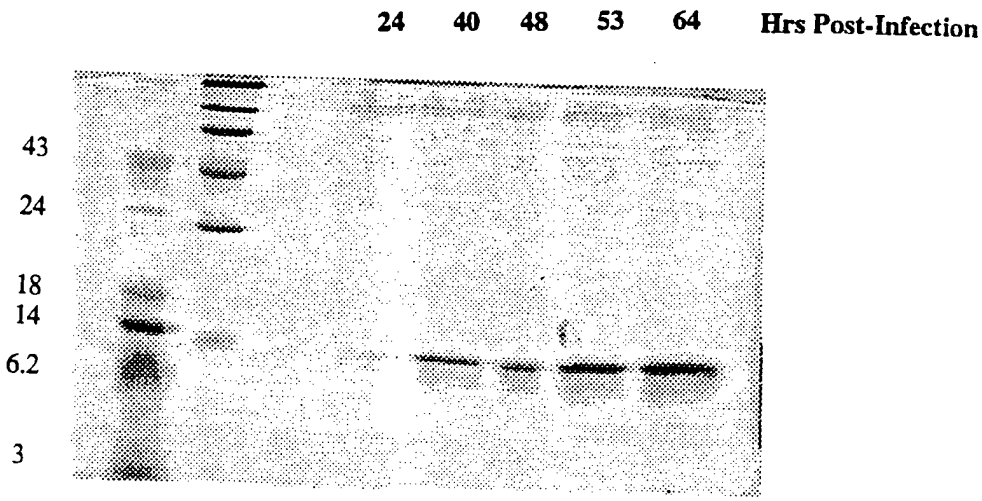
B) Immunoblot of P123.COCO using polyclonal anti-human TSP1 antibody. Samples were denatured with SDS, reduced with BME, and boiled for 10 minutes prior to loading on a 14% SDS-PAGE. The proteins were transferred to nitrocellulose blocked with non-fat milk, and probed with rabbit anti-hTSP1 Ab. ECL detection was used. RM, Rainbow molecular weight markers; CM, Conditioned media from P123.COCO infected cells; P123, purified P123.COCO.

Figure 7

A) MOI of 2

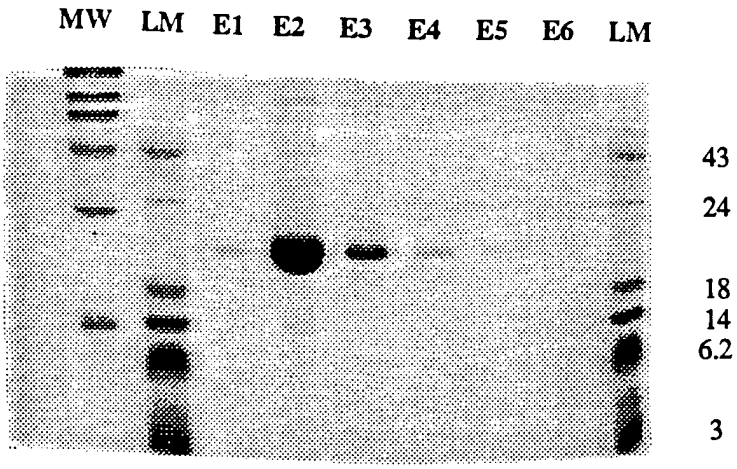


B) MOI of 5



Time course of infection of P3.COCO expressed in High Five Cells. Coomassie blue stain of P3.COCO samples after elution from Ni-NTA resin. For each time point 1ml aliquot was purified on NiNTA resin. Elution Buffer 20mM Tris-Cl, 150mM NaCl, 250mM Imidazole, pH 7.4. The samples were denatured with SDS, reduced with BME, and boiled for 5 minutes prior to loading on 15% SDS-PAGE. MW, Molecular weight markers; LM, Low molecular weight markers; Time Points post-infection: 24hrs, 40hrs, 48, 53, 64. A) Multiplicity of Infection (MOI) of 2 B) MOI of 5.

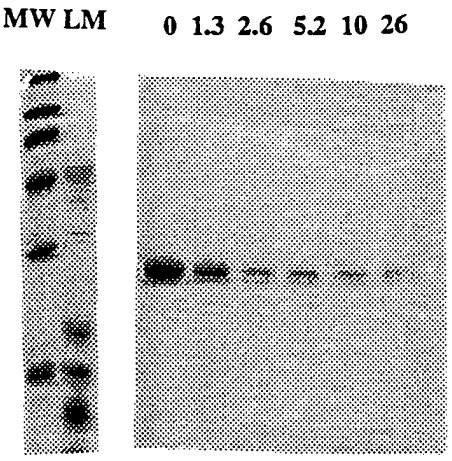
Figure 8

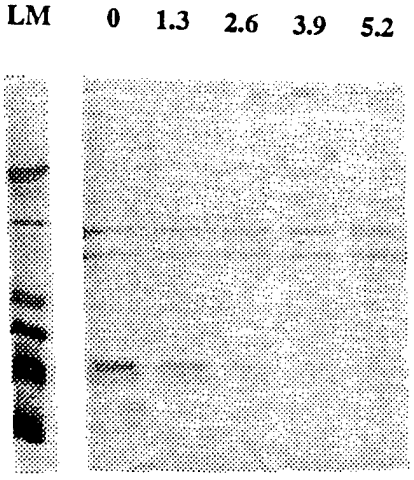


Coomassie blue stain of P123.COCO after elution from Ni-NTA column. Elution Buffer 20mM Tris-Cl, 150mM NaCl, 250mM Imidazole, pH 7.4. Fractions were collected by single column volumes (E1 through E6). For each eluate fraction, 5ul of sample was denatured with SDS, reduced with BME, and boiled for 5 minutes prior to loading on a 14% SDS-PAGE. MW, Molecular weight markers; LM, Low molecular weight markers; E1, first eluate fraction; E2, second eluate fraction; etc.

Figure 9

Post-Thrombin, Bound to NiNTA





Digestion of P123.COCO (A) and P3.COCO (B) with Biotinylated Thrombin to remove His-tag. The reaction conditions were 50mMTris-Cl, 150mM NaCl, 2.5mM $CaCl_2$, pH 8.5 at 22C for ~20hrs. The samples were then incubated with NiNTA and eluted using TBS, 400mM Imidazole. The samples were denatured with SDS, reduced with BME, and boiled for 5 minutes prior to loading. MW, Molecular weight markers; LM, Low molecular weight markers.

Figure 10

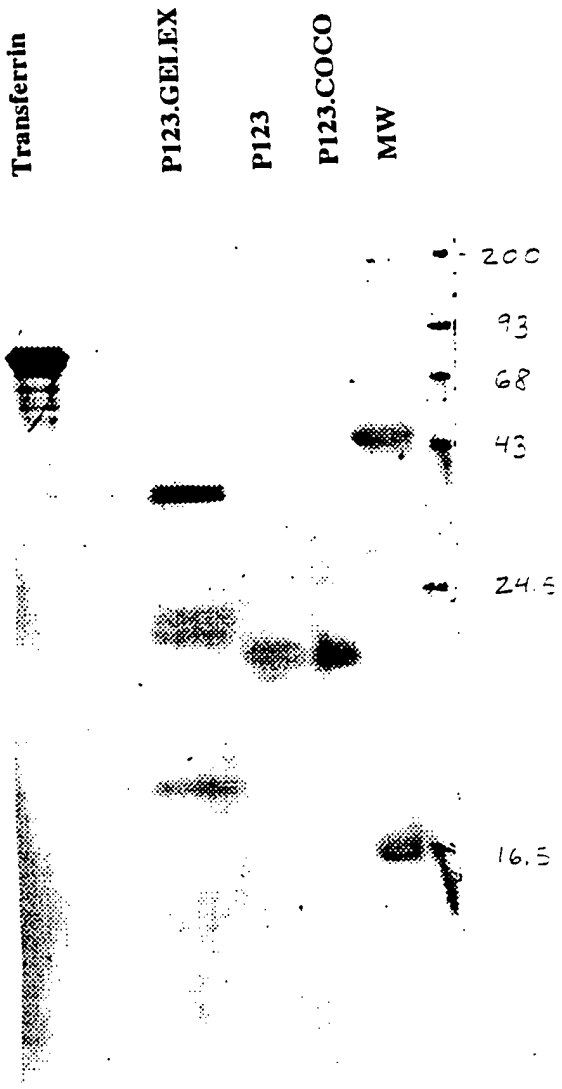
Amino Acid Sequence of P123.COCO including the GP67 signal sequence

```
     |----------Proposed GP67 signal sequence----------|
  1  MLLVNQSHQGFNKEHTSKMVSAIVLYVLLAAAAHSAFAADPGSDSADDGW
 51  SPWSEWTSCSTSCGNGIQQRGRSCDSLNNRCEGSSVQTRTCHIQECDKRF
101  KQDGGWSHWSPWSSCSVTCGDGVITRIRLCNSPSPQMNGKPCEGEARETK
151  ACKKDACPINGGWGPWSPWDICSVTCGGGVQKRSRLCNNPTPQFGGKDCV
201  GDVTENQICNKQDCPILELVPRGSAAGHHHHHHZ
```

N-TERMINAL SEQUENCE ANALYSIS OF P123.COCO

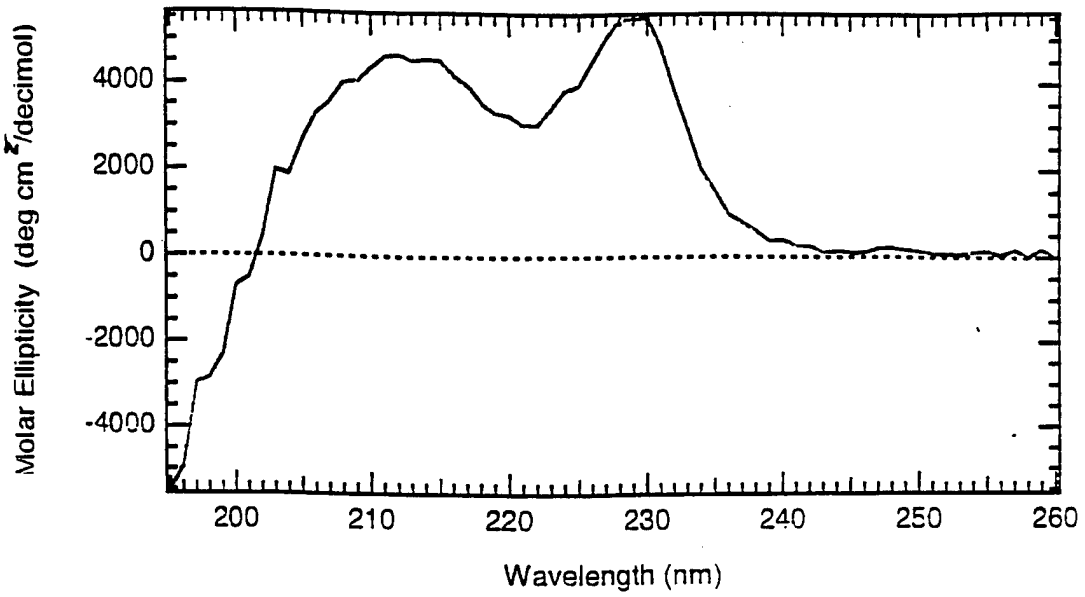
Cycle	First	Second	Match	Known
1	A	S	+++	A
2	D	P	+++	D
3	P		+++	P
4	G	S	+++	G
5	S	R	+++	S
6	D	T	+++	D
7	S	R	+++	S
8	A	T	+++	A
9	D	K	+++	D
10	D	T	+++	D
11	G	Q	+++	G
12	G			W
13	S	T	+++	S
14	P	S	+++	P
15	P	T		W

Figure 11



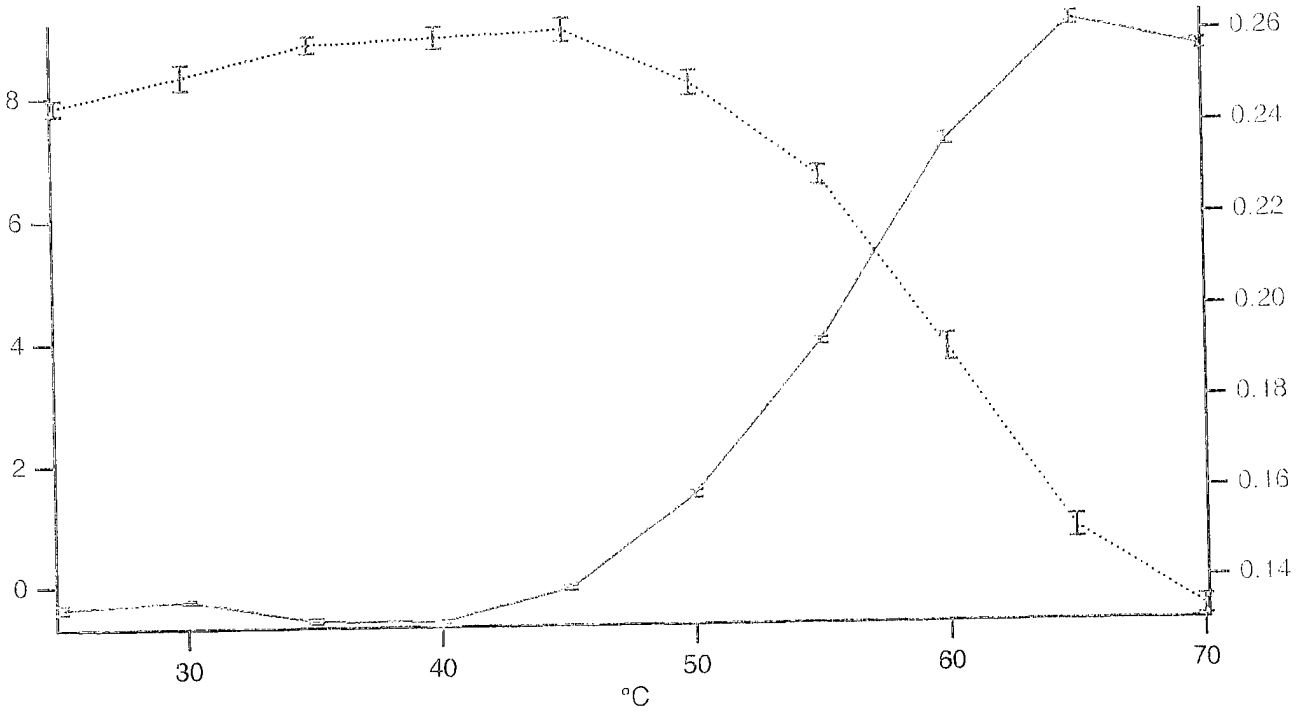
Immunoblot of P123.GELEX and P123.COCO using an anti-DIG-alkaline phosphatase conjugated antibody. Samples were denatured with SDS, reduced with BME, and boiled for 10 minutes prior to loading on a 14% SDS-PAGE. The proteins were transferred to nitrocellulose and DIG detection was performed according to manufacturer's instructions. +, Positive control glycoprotein Transferrin; P123.GELEX, trypsin digested; P123, post-thrombin; P123.COCO, purified; MW, Molecular weight markers.

Figure 12



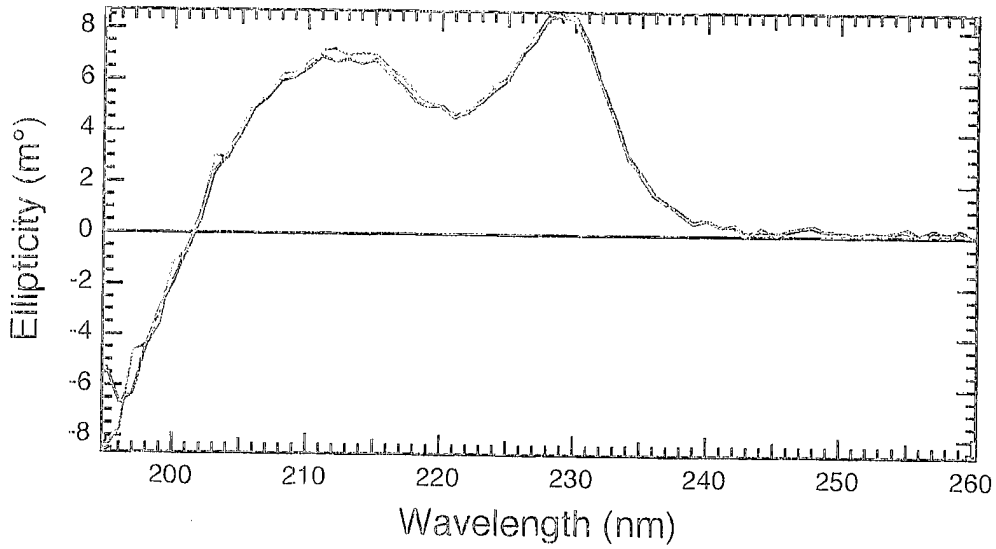
Circular Dichroism of P3: Far-Uv CD spectrum of the third type 1 repeat of hTSP1 in 0.1 cm pathlength cell, scanned from 260nm to 195nm.

Figure 13



Temperature Scan of P3. The Circular Dichroism signal was monitored at 229nm and the wavelength for excitation of fluoresence was 291nm. The CD signal is shown in red and the fluorescence signal is shown in blue.

Figure 14



Far-UV Circular Dichroism of P3 before and after heating. Far-UV CD spectrum of P3 in 0.1 cm pathlength cell, scanned from 260nm to 195nm.